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Phytoncides (Wood Essential Oils) Induce Human Natural Killer Cell Activity

Qing Li,[1] Ari Nakadai,[1] Hiroki Matsushima,[2] Yoshifumi Miyazaki,[3] Alan M. Krensky,[4] Tomoyuki Kawada,[1] and Kanehisa Morimoto[2]

[1]Department of Hygiene and Public Health, Nippon Medical School, Tokyo, Japan
[2]Department of Social and Environmental Medicine, Osaka University Graduate School of Medicine, Osaka, Japan
[3]Forestry and Forest Products Research Institute, Tsukuba city, Ibaraki Prefecture, Japan
[4]Division of Immunology and Transplantation Biology, Stanford University School of Medicine, Stanford, California, USA

To explore the effect of forest bathing on the human immune system, we investigated the effect of phytoncides (wood essential oils) on natural killer (NK) activity and the expression of perforin, granzyme A and granulysin in human NK cells. We used NK-92MI cell, an interleukin-2 independent human NK cell line derived from the NK-92 cell, in the present study. NK-92MI cells express the CD56 surface marker, perforin, granzyme A, and granulysin by flow cytometry and are highly cytotoxic to K562 cells in chromium release assay. Phytoncides significantly increase cytolytic activity of NK-92MI cells in a dose-dependent manner and significantly increase the expression of perforin, granzyme A, and granulysin in the NK-92MI cells. Phytoncides also partially, but significantly, restore the decreased human NK activity and the decreased perforin, granzyme A, and granulysin expression in NK-92MI cells induced by dimethyl 2,2-dichlorovinyl phosphate (DDVP), an organophosphorus pesticide. Pretreatment with phytoncides partially prevents DDVP-induced inhibition of NK activity. Taken together, these data indicate that phytoncides significantly enhance human NK activity and this effect is at least partially mediated by induction of intracellular perforin, granzyme A, and granulysin.

Keywords Flow Cytometry, Granulysin, Granzyme A, NK Activity, Perforin, Phytoncides.

Address correspondence to Qing Li, Department of Hygiene and Public Health, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan; E-mail: qing-li @nms.ac.jp (Q.Li)

INTRODUCTION

Exposure to essential oils/fragrances from trees or fruits has been reported to improve anxious or depressive status, reduce the sympathetic activity under stress, and reduce blood pressure in humans or animals.[1–3] Aoshima and Hamamoto [4] reported that perfume and phytoncide have an effect on $GABA_A$ receptors expressed in *Xenopus* oocytes. Moreover, human endocrine and immune systems are affected by fragrance as determined by analysis of urinary cortisol and dopamine levels, natural killer (NK) activity, and CD4/8 ratios.[5] Shibata et al. [6] further reported that fragrance exposure has a restorative effect on the stress-induced immune suppression in mice. However, there have been no reports investigating the effect of phytoncytes on human NK function *in vitro*.

NK, lymphokine-activated killer (LAK), and cytotoxic T lymphocyte (CTL) cells induce tumor- or virus infected target cell death by two main mechanisms.[7–9] The first mechanism is the direct release of cytolytic granules that contain the pore-forming protein perforin, several serine proteases termed granzymes,[10–13] and granulysin [14–16] by exocytosis to kill target cells. The second mechanism is mediated by the Fas ligand (FasL)/Fas pathway in which FasL (CD95L) a surface membrane ligand of the killer cell cross-links with the target cell's surface death receptor Fas (CD95) to induce apoptosis of the target cells.[9,17–19]

To test the effect of phytoncides on human NK cell function, we investigated whether phytoncides affect NK activity and the expression of perforin, granzyme, and granulysin in human NK cells.

NK-92 is an interleukin-2 (IL-2)-dependent human NK cell line derived from peripheral blood mononuclear cells from a 50-year-old Caucasian male with rapidly progressive non-Hodgkin's lymphoma.[20] NK-92CI and NK-92MI are IL-2 independent NK cell lines derived from the NK-92 cell line by transfection of human IL-2 cDNA.[21] The cell lines are cytotoxic to a wide range of malignant cells, killing K562 cell in a chromium release assay.[22] NK-92 (and derivative cell lines) have the following characteristics: surface marker positive for CD2, CD7, CD11a, CD28, CD45, CD54, and CD56 bright; surface marker negative for CD1, CD3, CD4, CD5, CD8, CD10, CD14, CD16, CD19, CD20, CD23, CD34, and HLA-DR.[21] We also previously showed that NK-92CI and NK-92MI cells express high levels of CD56, perforin, granzyme A, and granulysin.[22]

Therefore, we used NK-92MI cell line to investigate the effect of phytoncides on NK activity and the intracellular levels of perforin, granzyme A and granulysin in the human NK cells.

MATERIALS AND METHODS

Reagents

Phytoncides, the wood essential oil such as Chamaecyparis (hinoki) stem oil, Chamaecyparis leaf oil, white cedar (hiba) stem oil, Japanese cedar

(cryptomeria, sugi) stem oil, Chamaecyparis taiwanensis stem oil, α-pinene, 1,8-cineole, and d-limonene were purchased from Wako Pure Chemical Industries (Osaka, Japan). Alpha minimum essential medium (α-MEM) without ribonucleosides and deoxyribonucleosides, inositol, 2-mercaptoethanol (2-ME), folic acid, glutamine, and propidium iodide (PI) were obtained from Sigma (St. Louis, MO, USA). Horse serum and fetal bovine serum (FBS) for NK-92CI and NK-92MI cell lines culture were purchased from American type culture collection (ATCC) (Manassas, VA, USA). RPMI 1640 medium was purchased from Nissui Pharmaceutical (Tokyo, Japan). FBS for K-562 culture was purchased from JRH Biosciences (Lenexa, KS, USA) and heat-inactivated at 56 °C for 30 min prior to use. Dimethyl 2,2-dichlorovinyl phosphate (DDVP) and Triton X-100 were purchased from Wako Pure Chemical Industries (Osaka, Japan). Sodium ^{51}Cr-chromate was obtained from PerkinElmer (Boston, MA, USA). Fluorescein isothiocynate (FITC)-mouse antihuman perforin (IgG2b), granzyme A (IgG1), and phycoerythrin (PE)-CD56 (IgG1), FITC/PE-negative isotypic control antibodies, and Cytofix/cytoperm solution were purchased from BD Pharmingen (San Diego, CA, USA). Rabbit antihuman granulysin polyclonal antibody was described previously.[23] PE- goat-antirabbit IgG were purchased from Vector Laboratories (Burlingame, CA, USA).

Cells

NK-92MI cell line, obtained from ATCC, was maintained in α-MEM without ribonucleosides and deoxyribonucleosides with 2 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and supplemented with 0.2 mM inositol, 0.1 mM 2-ME, 0.02 mM folic acid, 12.5% horse serum, and 12.5% FBS at 37 °C in 5% CO_2 incubator. K-562 target cell also was obtained from ATCC and was maintained in RPMI 1640 medium containing 10% FBS.

Cytotoxicity of Phytoncides

The nonspecific cytotoxicity of phytoncides against NK-92MI cells was examined to determine the optimal concentrations for the following NK activity assay and flow cytometric analysis, where the phytoncides do not kill NK-92MI cells but can activate the NK cells. NK-92MI cells at 1–2 × 10^5/ml were incubated with phytoncides at 0, 0.1, 0.2, 1, 10, 100, 500, 1000, and 5000 ppm for 8–144 hr at 37 °C in 5% CO_2 incubator, then harvested, and washed with PBS. The dead cells were stained with PI and analyzed by flow cytometry and the viability was calculated.

Treatment with Phytoncides and/or DDVP

NK-92MI cells were treated by the following 3 different ways:

1. NK-92MI cells at 1×10^5/ml were incubated with phytoncides at 0, 0.05, 0.1ppm for 24–144 hr at 37 °C in 5% CO_2 incubator, and then harvested, washed with α-MEM, and used for the subsequent NK activity assay and flow cytometric analysis.
2. NK-92MI cells at 1×10^5/ml were incubated with phytoncides at 0, 0.05, 0.1 ppm for 72–120 hr at 37 °C in 5% CO_2 incubator, and then harvested, washed with α-MEM, and then treated with DDVP at 20–80 ppm for 15 hr at 37 °C in 5% CO_2 incubator, and then harvested, washed with α-MEM, and were used for the subsequent NK activity assay.
3. NK-92MI cells at 2×10^5/ml were preincubated with DDVP at 6.25 ppm for 15 hr at 37 °C in 5% CO_2 incubator, and then harvested, washed with α-MEM, and treated with phytoncides at 0, 0.1 ppm for 48–120 hr at 37 °C in 5% CO_2 incubator, then harvested, washed with α-MEM, and used for the subsequent NK activity assay and flow cytometric analysis.

NK Activity Assay

A standard microtiter ^{51}Cr-release assay was used to measure NK activity as described previously.[13,19,24] Briefly, K-562 target cells were labeled with sodium ^{51}Cr-chromate solution for 60 min at 37 °C in 5% CO_2 and washed 4 times in RPMI 1640 containing 10% FBS. The target cells were plated into round-bottomed 96-well microplates, and then the NK-92MI cells after treatment with phytoncides and/or DDVP were added to the wells in triplicate at indicated effector/target (E/T) ratios. Following a 4-hr incubation, the microplates were centrifuged, and 0.1 ml of supernatant from each well was collected and counted in a gamma counter. The cytolytic activity induced by NK-92MI was calculated by averaging cpm for triplicate wells as described previously.[24]

Cell Staining and Flow Cytometric Analysis

The NK-92MI cells were fixed/permeablized with cytofix/cytoperm solution for 20 min at 4 °C, and then the intracellular perforin and granzyme A were stained with FITC- antihuman perforin and grranzyme A, respectively, for 30 min at 4 °C, according to the manufacturer's instructions (BD PharMingen). Intracellular granulysin was stained with rabbit antihuman granulusin polyclonal antibody after fixation/permeablization with cytofix/cytoperm solution, and then stained with PE-goat antirabbit IgG for 30 min at 4 °C in the dark.

After staining, the cells were washed twice with the fix washing and once with PBS containing 1% FBS. Flow cytometric analysis was performed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) as described previously.[22] NK-92MI cells were identified by their characteristic appearance on a dot plot of FSC versus SSC and electronically gated to exclude dead cells.

Statistical Analysis

Multiple comparisons were made with Tukey's method if the analysis of variance was significant. The analysis was performed with the SPSS 11.5J software package for Windows. The significance level for p values was set at < 0.05.

RESULTS

Cytotoxicity of Phytoncides

Phytoncides at 1 ppm or more were toxic to NK-92MI cells, whereas at 0.2 ppm, none of the phytoncides showed cytotoxicity. Based on these results, 0.05 and 0.1 ppm of phytoncides were selected for the subsequent NK activity assay and flow cytometric analysis.

Effect of Phytoncides

Chamaecyparis (hinoki) stem oil, white cedar (hiba) stem oil, 1,8-cineole, and α-pinene significantly increased NK activity of NK-92MI in a dose-dependent manner after 120 hr (Fig. 1A) and 144 hr (Fig. 1B) of *in vitro* incubation except with 1,8-cineole at 120 hr. There was no effect for 24–96 hr of *in vitro* incubations, and 144 hr of *in vitro* incubation showed a stronger effect than 120 hr *in vitro* incubation (Figs. 1A and 1B), indicating that this effect was time-dependent. We confirmed that phytoncides at the highest concentration used in the present study did not show nonspecific cytotoxicity against NK-92MI after 144 hr culture *in vitro* as determined by flow cytometry.

Pretreatment of NK-92MI with Phytoncides

Pretreated NK92MI cells with Chamaecyparis (hinoki) stem oil and white cedar (hiba) stem oil for 72 hr (Fig. 2A), 96 hr (Fig. 2B), and 120 hr (Fig. 2C) significantly increased the resistance of NK-92MI to DDVP in a dose-dependent manner. In contrast, 1,8-cineole and α-pinene showed no protection from DDVP after 72 hr pretreatment (Fig. 2A). Protection did occur after pretreatment for 96 hr (Fig. 2B) and 120 hr (Fig. 2C), respectively, indicating that this preventive effect was time-dependent.

Phytoncides Partially Restore DDVP-Induced NK Activity Inhibition

As shown in Fig. 3, DDVP induced a decrease of NK activity of NK-92 cells. However, phytoncides at 0.1ppm partially, but significantly, restored the DDVP-induced NK activity inhibition except with 1.8-cineole.

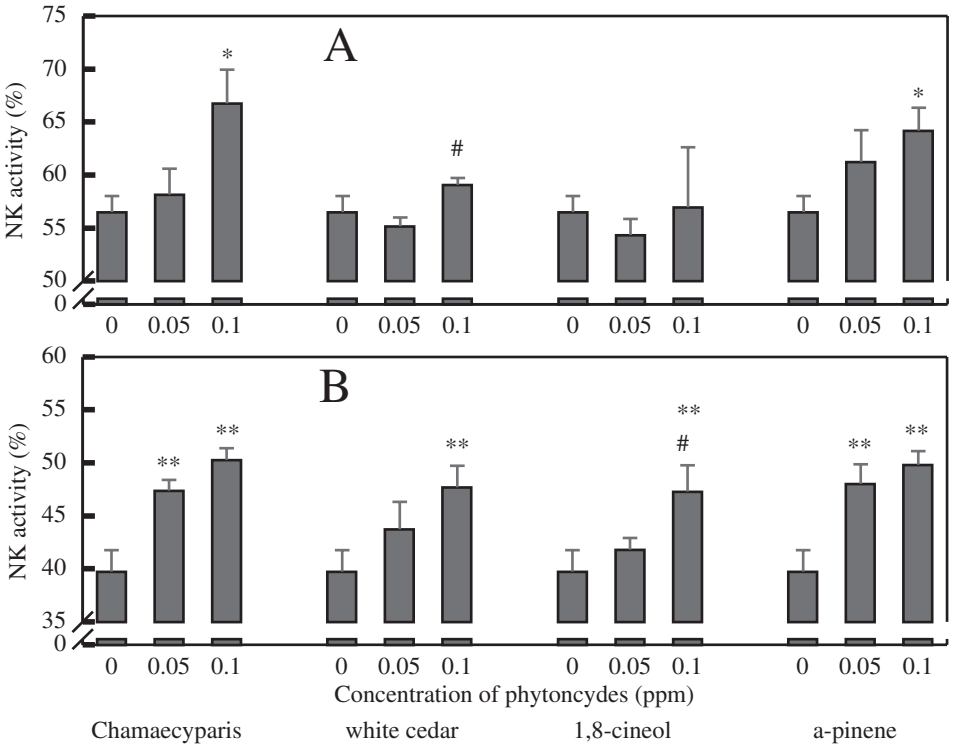

Figure 1: Effect of phytoncydes on NK activity of NK-92MI cells after incubations of 120 hr (A) and 144 hr (B). Data are presented as the mean ± SD (n = 3). One-way ANOVA analysis indicated that the doses of 4 kinds of phytoncydes significantly affected the NK activity for 120 hr (A) and 144 hr (B) (all $p < 0.05$) except with 1,8-cineole at 120 hr. *$p < 0.05$, **$p < 0.01$, significantly different from 0 ppm, #$p < 0.05$ significantly different from 0.05 ppm by Tukey's method. The activity values for an E/T ratio of 0.5/1 are shown, and similar results also were obtained with E/T ratios of 0.25/1 and 1/1.

Expression of Perforin, Granzyme A, and Granulysin

The fluorescent intensity of FITC-perforin/granzyme A and PE-granulysin was used to measure the expression of perforin, granzyme A, and granulysin in NK-92MI cells. As shown in Figures 4, 5, and 6, treatment with phytoncides for 120 hr induced a marked increase in expression of granzyme A (Figure 4A), granulysin (Figure 5A), and perforin (Figure 6A). All the phytoncides markedly increased expression of granzyme A (Figure 4B), granulysin (Figure 5B), and perforin (Figure 6B).

Phytoncides Partially Restore the DDVP-induced Decrease of Granzyme A and Granulysin

We previously showed that DDVP decreased the levels of granzyme A, granulysin, and perforin in NK-92 cells.[22] In the present study, we pretreated

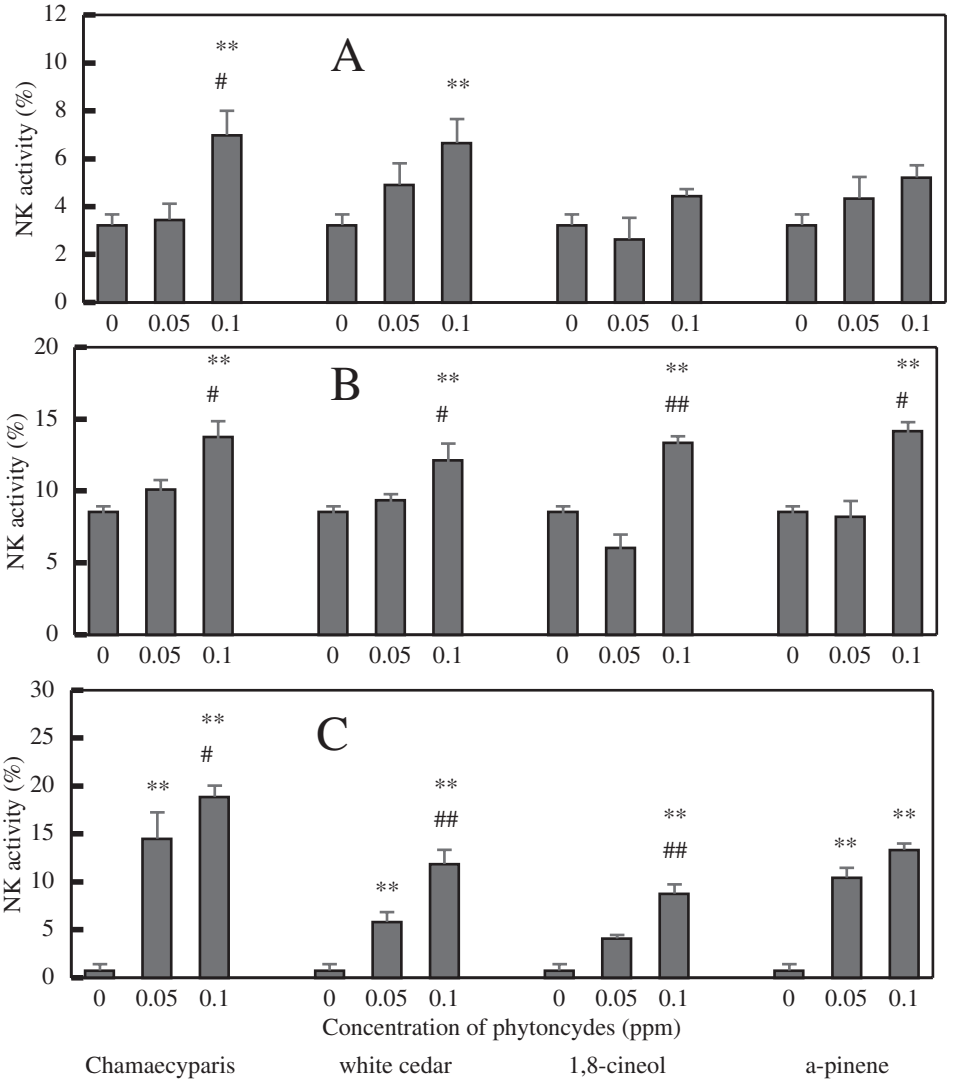

Figure 2: Pretreatment of NK-92MI with phytoncides increases the resistance of NK-92MI to DDVP toxicity. NK-92MI cells at 1×10^5/ml were incubated with phytoncides at 0, 0.05, and 0.1 ppm for 72 hr (A), 96 hr (B), and 120 h (C) at 37 °C in 5% CO_2 incubator, then harvested, washed with α-MEM, treated with DDVP at 20–80 ppm for 15 hr at 37 °C in 5% CO_2, harvested, washed with α-MEM, and the NK activity was measured. Data are presented as the mean ± SD (n = 3). One-way ANOVA analysis indicated that the dose of phytoncides significantly affected the NK activity (all $p < 0.01$) except with 1.8-cineole and α-pinene at 72 hr (A). *$p <$ 0.05, **$p < 0.01$, significantly different from 0 ppm, #$p < 0.05$, ##$p < 0.01$ significantly different from 0.05 ppm by Tukey's method.

NK-92 cells with DDVP and then incubated the cells with phytoncides to investigate whether phytoncides can restore the DDVP-induced decreases of granzyme A and granulysin in NK-92 cells. As shown in Figure 7, treatment with Chamaecyparis (hinoki) stem oil for 72 hr induced a marked increase in

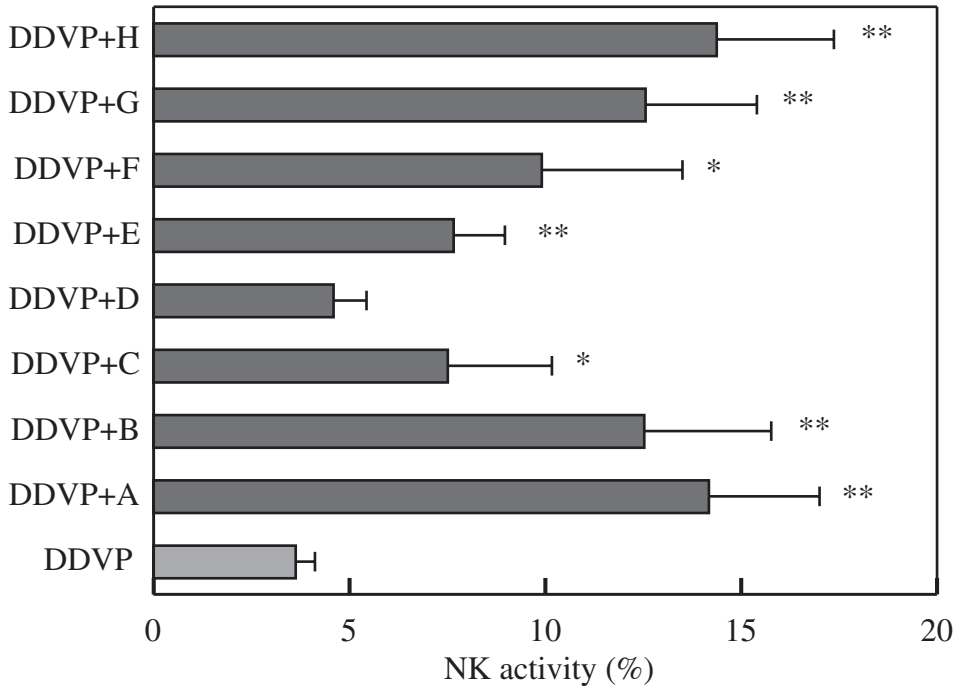

Figure 3: Phytoncides partially reverse DDVP-induced inhibition of NK activity. NK-92MI at 2×10^5/ml were preincubated with DDVP at 6.25 ppm for 15 hr at 37°C in 5% CO_2 incubator, then harvested, washed with α-MEM, and treated with phytoncides at 0 and 0.1 ppm for 72 hr at 37°C in 5% CO_2 incubator, harvested, washed with α-MEM, and the NK activity was measured. A-chamaecyparis (hinoki) stem oil, B-chamaecyparis leaf oil, C-white cedar (hiba) stem oil, D-1,8-cineole, E-chamaecyparis taiwanensis stem oil, F-Japanese cedar (cryptomeria; sugi) stem oil, G-α-pinene, H-d-limonene. Data are presented as the mean ± SD (n = 3). One-way ANOVA analysis indicated a significant difference among the treatments ($p < 0.01$). *$p < 0.05$, **$p < 0.01$, significantly different from DDVP alone by Tukey's method.

expression of granzyme A (Figure 7A) and granulysin (Figure 7B) compared with treatment with DDVP alone. This indicates that Chamaecyparis (hinoki) stem oil could partially restore the DDVP-induced decrease of granzyme A and granulysin in NK-92MI cells. Other phytoncides have similar effects (not shown).

DISCUSSION

The present study demonstrated that phytoncides can enhance human NK activity *in vitro* and that this effect is dose- and time-dependent. This is the first report to investigate the direct effect of phytoncides on human NK activity *in vitro*, although Komori et al.[5] and Shibata et al.[6] have reported that fragrance from trees has a regulatory effect on immune function in humans and a restorative effect on the stress-induced immune suppression in mice, respectively.

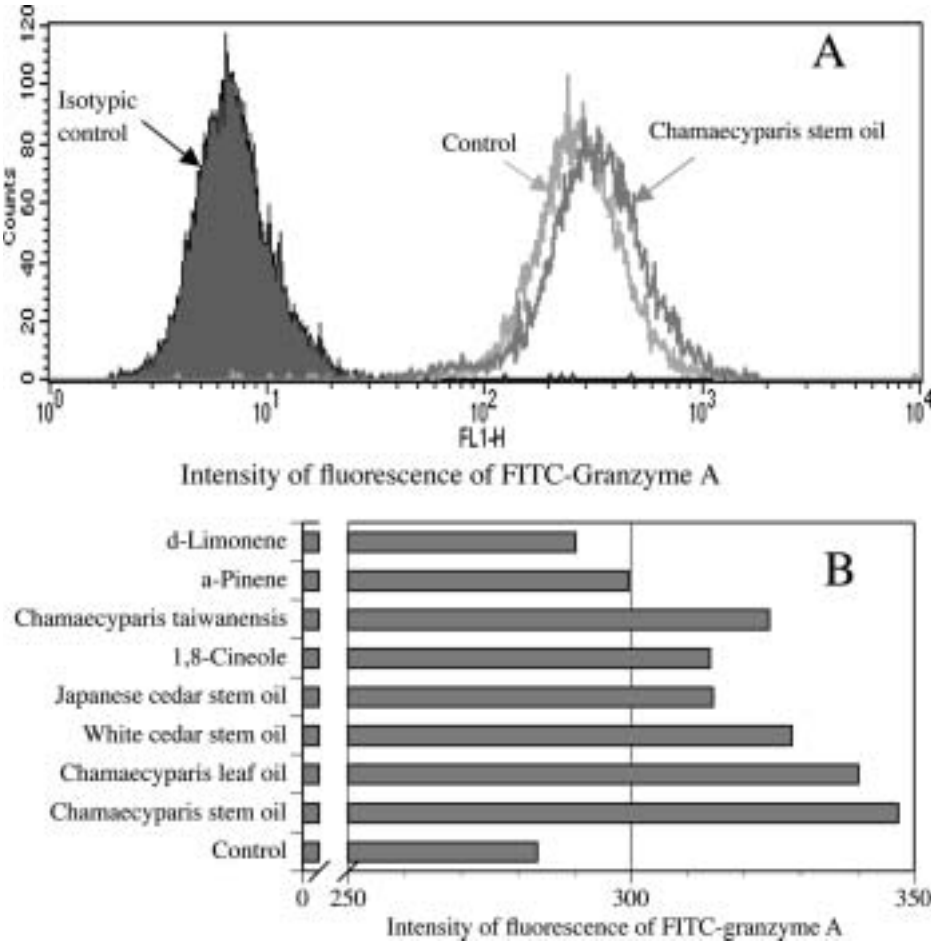

Figure 4: Effect of phytoncides on the expression of granzyme A in NK-92MI after 120 hr *in vitro* treatment. (A) The solid histograms show the control stained with FITC-mouse IgG1 (isotype control); the histograms with the gray line show the results stained with FITC-mouse antihuman GrA in cells without treatment of phytoncide (control), and the histograms with dark gray line show the results stained with FITC-mouse antihuman granzyme A in cells after treatment with Chamaecyparis (hinoki) stem oil at 0.1 ppm. (B) The fluorescent intensity of FITC-antigranzyme A in NK-92MI among different phytoncides. All experiments were repeated at least 3 times with similar results.

As shown in Fig. 1, because NK-92 cells have a very strong cytolytic activity, it is difficult to enhance the NK activity further. Therefore, the effect of phytoncides on the cytolytic activity of NK-92 cells could be underestimated. To overcome this obstacle, we reduced the cytolytic activity of NK-92 cells by immunotoxic substances and then investigated the effect of phytoncides on restoration of cytolytic activity of NK-92 cells. Since we previously found that DDVP significantly inhibits NK activity *in vitro*, [13,22,24] in the present study, we reduced the cytolytic activity of NK-92 cells with DDVP and then treated

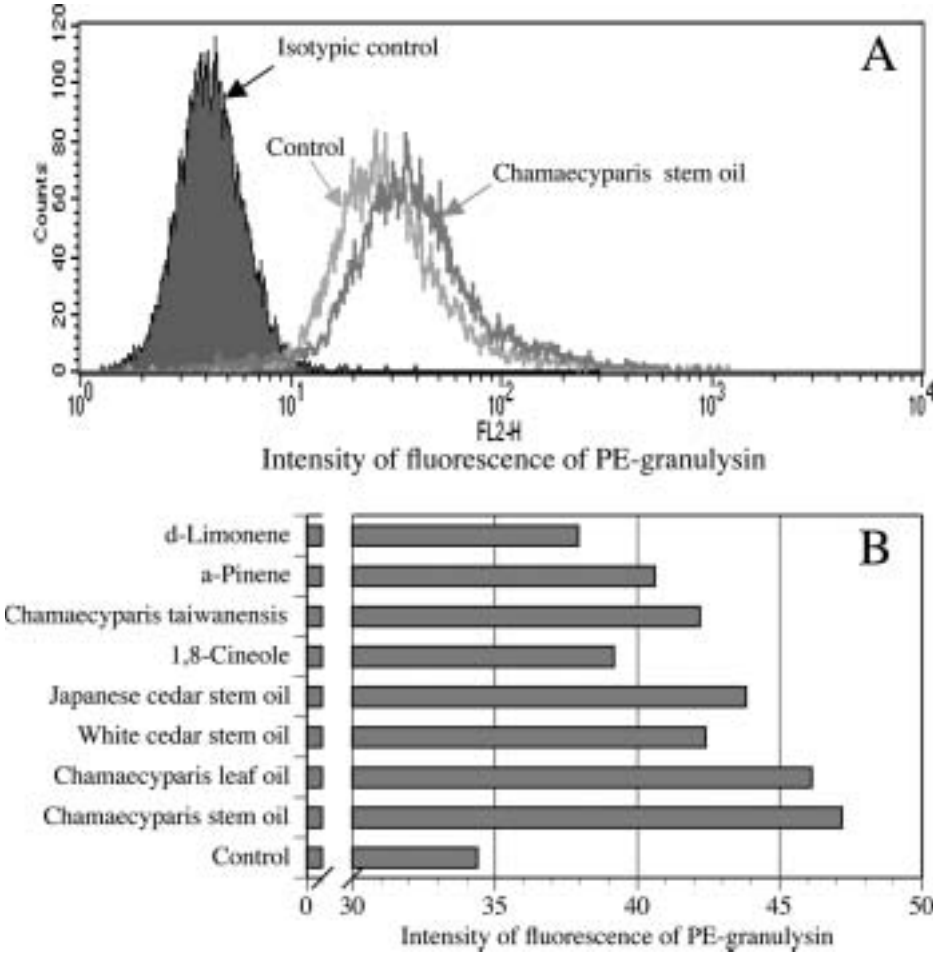

Figure 5: Effect of phytoncides on the expression of granulysin in NK-92MI after 120 hr of *in vitro* treatment. (A) The solid histograms show the control stained with PE-antirabbit IgG (isotype control); the histograms with the gray line show the results stained with PE-antihuman granulysin in cells without treatment of phytoncide (control), and the histograms with dark gray line show the results with PE-antihuman granulysin in cells after treatment with Chamaecyparis (hinoki) stem oil at 0.1 ppm. (B) The fluorescent intensity of PE-antihuman granulysin in NK-92MI among different phytoncides. All experiments were repeated at least 3 times with similar results.

the NK-92 cells with the phytoncides to investigate the effect of phytoncides on the reduced NK activity. We found that phytoncides partially, but significantly, restored DDVP-induced inhibition of cytolytic activity of NK-92 cells. Next, we asked whether phytoncides could increase the resistance of NK cells to DDVP. The present findings indicate that pretreatment with phytoncides significantly increased the resistance of NK cells to DDVP, suggesting that phytoncides have a prophylactic preventive effect on NK cells against at least some immunotoxic substances.

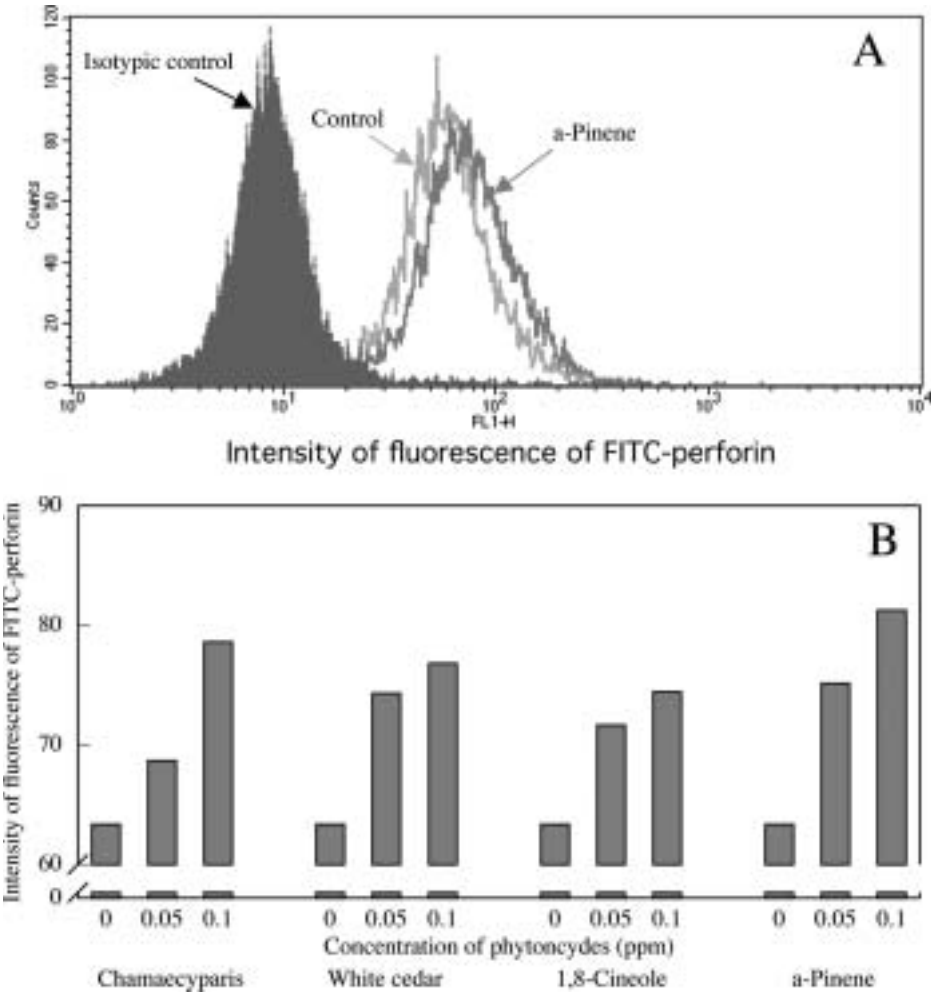

Figure 6: Effect of phytoncides on the expression of perforin in NK-92MI after 120 h of *in vitro* treatment. (A) The solid histograms show the control stained with FITC-mouse IgG2b (isotype control); the histograms with the gray line show the results stained with FITC-mouse antihuman perforin in cells without treatment of phytoncide (control), and the histograms with dark gray line show the results with FITC-mouse antihuman perforin in cells after treatment with α-pinene at 0.1ppm. (B) The fluorescence intensity of FITC-antiperforin in NK-92MI among different phytoncides. All experiments were repeated at least 3 times with similar results.

NK cells kill tumor- or virus-infected cells by release of perforin, granzymes,[11,12] and granulysin[15,16] *via* the granule exocytosis pathway. Cytotoxicity mediated by NK cells is greatly impaired in perforin-deficient mice.[17,19] Granzyme A plays a critical role in triggering apoptosis in target cells either directly or *via* the activation of cellular caspases, and it also cleaves IL-1β, the nucleosome assembly protein called putative HLA-associated protein II, TAF-Iβ, histones, and lamins.[12,25,26] Granulysin, a lytic

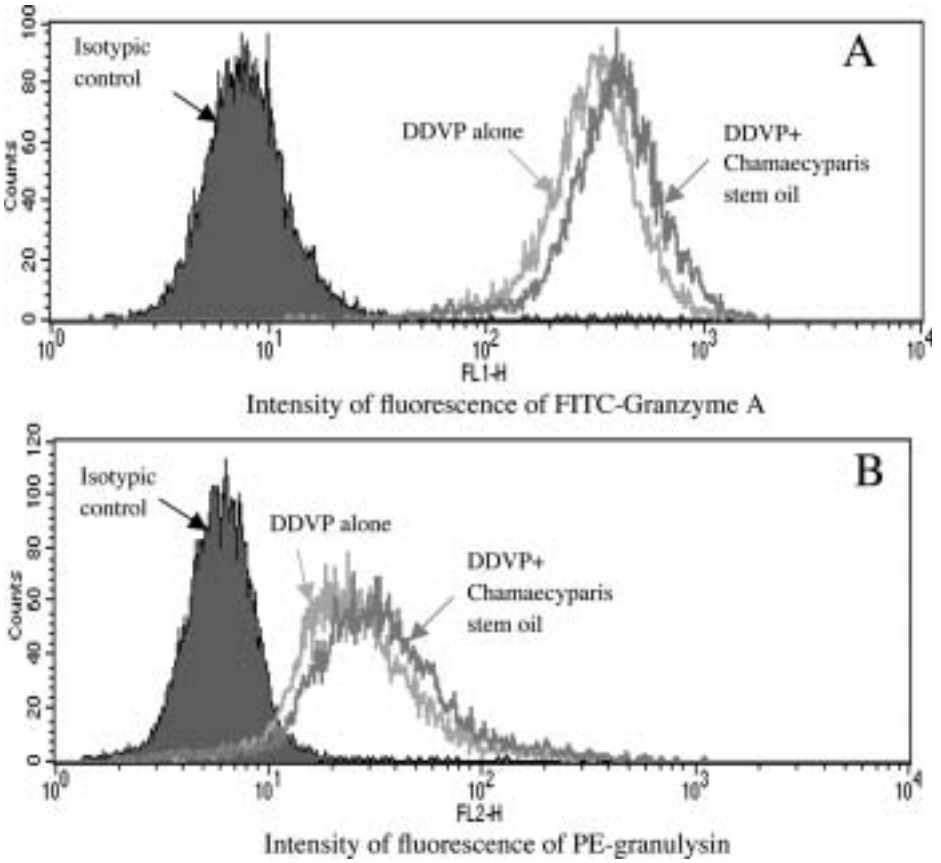

Figure 7: Phytoncides can partially restore the DDVP-induced decreases of granzyme A (A) and granulysin (B). The solid histograms show the control stained with FITC-mouse IgG1 (granzyme A; A) or PE-antirabbit IgG (granulysin; B) (isotype control); the histograms with gray lines show the results for FITC-mouse antihuman granzyme A (A) or PE-antihuman granulysin (B) in cells treated with DDVP alone. The histograms with dark gray lines show the results in the cells treated with DDVP, and then with Chamaecyparis (hinoki) stem oil at 0.1 ppm for 72 hr. All experiments were repeated at least 3 times with similar results.

molecule expressed by human CTL and NK cells, is active against tumor cells and a variety of microbes. Granulysin can enter target cells in the absence of perforin and induce apoptosis, although granulysin and perforin together are required to kill intracellular microbes like *Mycobacteria tuberculosis*.[15,16,27] Granulysin is associated with diverse activities of NK cells and CTL in physiological and pathological settings and may be a useful marker to evaluate the status of host cellular immunity.[28]

To explore the mechanism of the phytoncide-induced enhancement of human NK activity, we investigated the effect of phytoncides on perforin, granzymes A, and granulysin expression in NK cells. We found that phytoncides significantly increased intracellular levels of perforin, granzyme A, and granulysin directly,

indicating that phytoncides increased NK activity at least partially *via* the increase in the intracellular levels of these three cytolytic molecules. Since we previously showed that DDVP significantly decreases intracellular granzyme A, perforin, and granulysin,[22] we further investigated the effect of phytoncides on DDVP-induced decrease of these cytolytic molecules. Phytoncides partially restored the DDVP-induced decreases in granzyme A and granulysin.

Taken together, phytoncides significantly enhance human NK activity and this effect at least partially is the result of increased expression of intracellular cytolytic molecules, perforin, granzyme A, and granulysin. Although the underlying mechanism of phytoncide-induced increases in intracellular perforin, granzyme A, and granulysin requires further study, the present findings strongly suggest that forest bathing may have beneficial effects on human immune function. We are planning to investigate this effect in a field study.

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